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Research Article
Enzymatic Acidolysis of Fish Oil with Milk Fat Fatty Acids for the Synthesis of Structured Lipid

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Abstract

Background and Objective: Structured lipids (SLs) consisting of saturated fatty acids (SFAs) at the outside position (sn-1,3) and polyunsaturated fatty acids (PUFAs) at the sn-2 position have good nutritional values and high stabilities for oxidation. The objective of this research was to synthesize SLs containing SFAs at the sn-1,3 position and PUFAs at the sn-2 position by the enzymatic acidolysis of fish oil with milk fat fatty acids (MFFAs). **Materials and Methods:** Fish oil, containing high PUFAs, was combined with milk fat that was hydrolyzed to MFFAs through saponification with KOH followed by acidification with HCl and extraction with hexane. SLs were synthesized by acidolysis using a specific lipase from *Mucor miehei*. The factors of substrate ratio and reaction time were studied. The SLs were analyzed for the fatty acid compositions, acylglycerol profiles and positional distributions of the fatty acids at sn-2 and sn-1,3. **Results:** The increasing proportions of the MFFAs and the increase in the reaction time increased the incorporation of SFA into the SLs. An acidolysis ratio of fish oil to MFFAs of 1:3 and a reaction time of 6 h at 40°C resulted in a good SL, where EPA and DHA were incorporated at sn-2 at 13.20 and 12.85%, respectively and SFAs (capric, lauric, myristic, palmitic and stearic acids) at sn-1,3 at approximately 58.25%. The SL had an acylglycerol profile containing triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG) at 69.45, 22.32 and 8.23%, respectively. **Conclusion:** The optimum enzymatic acidolysis conditions for synthesizing the SL were a molar ratio of fish oil to MFFAs of 1:3 and a reaction time of 6 h at 40°C. The SL has the potential to fortify high-nutrition dairy products.

Key words: Polyunsaturated fatty acid, enzymatic acidolysis, fish oil, milk fat fatty acid, structured lipid

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Structured lipids (SLs) are lipids whose fatty acid compositions or positional distributions have been modified by chemically or enzymatically catalyzed reactions[1]. SLs are usually synthesized to obtain high nutritional values or good functional properties with desired characteristics[2,3]. SLs containing saturated fatty acids (SFAs) and medium chain fatty acids (MCFAs) in the outer position (sn-1,3) and polyunsaturated fatty acids (PUFAs) at the sn-2 position have good nutritional values and high oxidation stabilities. PUFAs, as 2-monoacylglycerols, are more readily absorbed than other PUFA derivatives[4]. MCFA residues are easily absorbed and SFAs protect fats/oils from oxidation when at sn-1,2[5].

Fish oil is rich in PUFAs, especially EPA and DHA. These fatty acids are distributed in fish oil at sn-1, sn-2 and sn-3. The unsaturated fatty acids found at sn-1,3 cause the fish oil to be easily oxidized and rancid. The PUFAs at sn-1,3 are also difficult to metabolize in the body. Furthermore, milk fat contains many MCFAs and SFAs. MCFAs and SFAs can be incorporated into fish oil, replacing the fatty acids at sn-1,3. Therefore, fish oil and milk fat have potential for the synthesis of the SLs with PUFAs at sn-2 and SFAs at sn-1,3. SLs can be synthesized by the enzymatic/chemical acidolysis or interesterification of lipids but enzymatic methods are preferred because the enzymes have a high specificity of biocatalytic activity and waste treatment is less of a problem[6].

Various methods for synthesizing specific SLs have been carried out, including the synthesis of SLs rich in n-3 PUFAs by the acidolysis of cod liver oil and caprylic acid[7] or the synthesis of SLs rich in DHA in position 2 by the acidolysis of tuna oil with caprylic acid[8]. In addition, Balcao and Malcata[9] synthesized highly unsaturated fatty acids in butterfat by the interesterification and the acidolysis of butterfat with oleic acid. Nadeem *et al.*[10] developed a functional fat from a butter oil and *Moringa oleifera* oil blend by the interesterification process. In the previous study, SLs were synthesized through the transesterification of fish oil and milk fat but the PUFAs at the sn-2 position were relatively low[11].

In this research, SLs were synthesized through the enzymatic acidolysis of fish oil with the free fatty acids from milk fat called milk fat fatty acids (MFFAs) using specific lipases of sn-1,3 from *Mucor miehei*. Factors such as the ratio of fish oil to MFFAs and the reaction time were studied. The evaluation was carried out on the incorporation of SFAs and PUFAs, the acylglycerol profile and the positional distribution of the fatty acid residues at sn-1,3 and sn-2 in the SLs. This study aimed to synthesize SLs containing SFAs at the sn-1,3 position and PUFAs at the sn-2 position by the enzymatic acidolysis of fish oil with milk fat fatty acids.

MATERIALS AND METHODS

Materials: The fish oil "Prince Gold® Alaska Deep Sea Fish Oil" was obtained from Prince of Peace® Enterprises, Inc, USA; milk fat was obtained from PT Fonterra Brand Indonesia. The lipase from *Mucor miehei* (Lipozyme®) and porcine pancreatic lipase were obtained from Sigma-Aldrich (St. Louis, MO, USA). The hexane, acetone, ethanol, petroleum ether, acetic acid and diethyl ether were obtained from Merck KGaA (Darmstadt, Germany).

Preparation of the MFFAs from milk fat: The preparation of the MFFAs was performed according to Kim and Hill[12]. Approximately 100 g of milk fat was added to 40 g of KOH in 100 mL of distilled water and 300 mL of ethanol. The reactant mixture was then refluxed for 1 h. The purification of the fatty acid was carried out by adding 200 mL of distilled water, while the unsaponified material was extracted using 300 mL of hexane. The aqueous layer containing the saponified material was acidified by 6 N HCl until a pH of 1.0 was reached. The bottom layer was separated using a separating funnel, while the top layer containing the free fatty acids was extracted with 200 mL of hexane and washed with 100 mL of distilled water. The hexane fraction containing the fatty acids was removed and the remaining solvents were separated by a rotary evaporator.

Enzymatic acidolysis of the fish oil with MFFAs at various molar ratios: The acidolysis of the fish oil with MFFAs was performed at various molar ratios of the fish oil to the MFFAs (1:1; 1:3; 1:5; 1:10; and 1:15) so that the reactant mixture was approximately 10 g. Hexane was added at 1.5 times the amount of the substrate and then the immobilized lipase from *Mucor miehei* was added at levels as much as 10% of the weight of the fish oil. The reaction was carried out in a closed Erlenmeyer flask in a shaker incubator at 40°C for 4 h at a speed of 120 rpm. The reaction mixture was then filtered to separate the lipase. The filtrate was then added to 20 mL of acetone and ethanol at a ratio of 1:1. The reaction mixture was titrated with 0.5 M KOH to neutralize the free fatty acids. The acylglycerols or SLs were extracted by 30 mL of hexane and the remaining solvent was evaporated by a rotary evaporator. Enzymatic acidolysis for the synthesis of SLs at various reaction times: A mixture of fish oil and MFFAs was prepared at a molar ratio of 1:3. Hexane was added at 1.5 times the amount of the substrate and then the immobilized lipase from *Mucor miehei* was added at levels as much as 10% of the weight of fish oil. The reaction was carried out in a closed Erlenmeyer flask in a

shaker incubator for various reaction times (2, 4, 6, 12 and 24 h) at 40°C and 120 rpm. The reaction mixture was then filtered to stop the reaction. The filtrate was then added to 20 mL of acetone and ethanol at a ratio of 1:1. The reaction mixture was titrated with 0.5 M KOH to neutralize the free fatty acids. The acylglycerol or SL was extracted by 30 mL of hexane and the remaining solvent was evaporated by rotary evaporator.

Analysis of the fatty acid composition: The fatty acid composition was analyzed using gas chromatography (GC) after the trans-methylation to fatty acid methyl esters (FAMEs). Approximately 200 µL of sample was methylated by the addition of 400 µL BF3-methanol complex and heated at 90°C for 2 h. The FAME residues were extracted with 500 µL hexane and then analyzed by a "Varian 450-GC" equipped with a WCOT fused-silica of CP-Sil 5 CB column according to Subroto *et al.*[11]. FAMEs (2 µL) were injected during the GC and further identified by comparing their retention times with the authentic standard. The quantitative composition was obtained according to the AOCS Official Method Ce 1-62[13].

Analysis of the acylglycerol profiles: The composition of acylglycerols (MAG, DAG and TAG) was analyzed by thin layer chromatography (TLC)[14,15]. The silica gel TLC plate was activated in the oven at 105°C for 2 h. The samples were applied to an activated TLC plate and developed by the mobile phase consisting of petroleum ether: diethyl ether: acetic acid (60:40:1 v/v/v). The TLC plate was then dried and visualized with iodine vapor so that the spots of the acylglycerol component were brown. The quantitative analysis was carried out with the TLC Scanner Camag 3 "dummy" (S/N 081124). The D2 lamp was used, the wavelength was set at 350 nm and the scan speed was set at 20 mm sec^{-1}. The TLC scanner was equipped with winCATS Planar Chromatography software to quantify the results.

Analysis of the positional distributions of fatty acids in the SLs: The positional distributions of fatty acids in the SLs were determined using porcine pancreatic lipase and reagents according to Torres *et al.*[16]. The reactant mixture was stirred at a speed of 300 rpm at 40°C for 7 min. The reaction was stopped by adding 1 mL of acetic acid (0.1 M). The mixture was then extracted with 3 mL chloroform. The solvent was evaporated and the reaction product was analyzed by TLC. The TLC plates were developed with petroleum ether : diethyl ether : acetic acid (60:40:1 v/v/v). The band/spot was visualized with iodine vapor. The corresponding 2-monoacylglycerol bands were scraped from the silica plate, extracted with 3 mL of hexane and then methylated as described above in the section on the GC analysis. These results provided information about the distribution of the fatty acid residues at the sn-2 position. The distribution of the fatty acid residues at the sn-1.3 position was then calculated by subtracting the amount of the fatty acid residues at the sn-2 position from the total amount of the fatty acids in the SLs as determined by GC and based on the following Zock *et al.*[17] equation (1).

$$\text{sn}-1,3(\%)=\frac{3(\%\text{of total})-(\%\text{of sn}-2)}{2} \quad (1)$$

RESULTS AND DISCUSSION

Fish oil and MFFAs characteristics: The fatty acid composition of the fish oil was dominated by PUFAs, including EPA (23.10%) and DHA (12.21%). The SFAs were relatively low at approximately 32.77%. The fatty acid composition of the fish oil was in accordance with Shahidi and Ambigaipalan[4] and Hita *et al.*[8] where the fish oil was dominated by PUFAs (EPA and DHA). The MFFA was dominated by SFAs, especially palmitic acid (39.53%), myristic acid (14.95) and stearic acid (12.49) (Table 1). The fatty acid composition of the milk fat was in accordance with Lubary *et al.*[18], where the milk fat was dominated by SFAs, especially palmitic acid and myristic acid. The fatty acid composition greatly determines the physical, chemical and nutritional properties of a fat/oil. PUFAs have good nutritional benefit when incorporated at sn-2, while SFAs can be metabolized quickly and can increase the stability of a fat/oil when they are at sn-1,3. Therefore, the process of the acidolysis of fish oil with MFFAs using a specific lipase from

Table 1: The fatty acid composition of fish oil and milk fat fatty acids (MFFAs)

Fatty acid	Fish oil (%)	MFFAs (%)
Capric, C10:0	ND	1.97±0.01
Lauric, C12:0	0.23±0.23	4.92±0.47
Miristic, C14:0	8.64±0.34	14.95±0.68
Palmitoleic, C16:1	13.42±1.11	1.80±0.02
Palmitic, C16:0	20.89±0.98	39.53±1.96
Linoleic, C18:2	4.83±0.40	ND
Oleic, C18:1	11.78±1.22	24.35±0.80
Stearic, C18:0	3.01±0.02	12.49±1.83
EPA, C20:5	23.10±2.91	ND
Eicosatetraenoic, C20:4	0.79±0.01	ND
Eicosenoic, C20:1	1.12±0.02	ND
DHA, C22:6	12.21±1.28	ND
∑ SFA	32.77	73.86
∑ PUFA	40.93	ND

Each value is presented as the mean±standard deviation (n = 2), ND: Not detected, SFA: Saturated fatty acid, PUFA: Polyunsaturated fatty acid, EPA: eicosapentaenoic acid, DHA: Docosahexaenoic acid

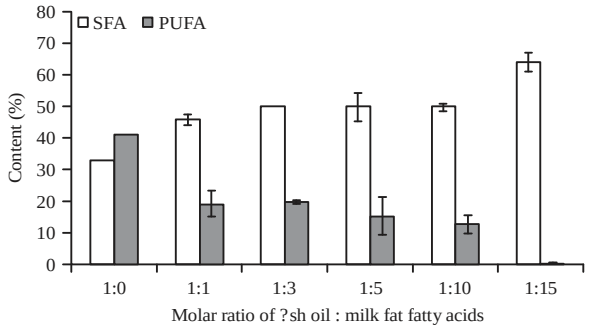

Fig. 1: The effect of the fish oil to MFFAs molar ratio on the SFA content and the PUFA content in the SLs

The reaction was performed using 10% immobilized lipase at 40°C for 4 h

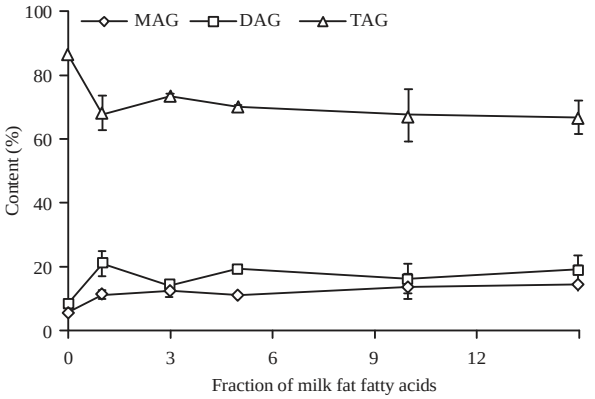

Fig. 2: The effect of the fish oil to MFFAs molar ratio on the TAG content, DAG content and MAG content in the SLs

The reaction was performed at 40°C for 4 h. For the x-axis, the number 1 describes a molar ratio of fish oil to MFFAs of 1:1, number 3 describes a ratio of 1:3 and so on

Mucor miehei was expected to produce SLs in which the sn-1,3 position contained the SFAs while the PUFAs remained at the sn-2 position.

Effect of the fish oil to MFFAs molar ratio on the SFA content and the PUFA content in the SLs: The fish oil contained PUFAs (EPA and DHA) at approximately 40.93% and SFAs, consisting of capric, lauric, myristic, palmitic and stearic acids, at approximately 32.77% (Table 1). The higher proportion of MFFAs increased the SFA content in the SLs. However, the PUFAs decreased, especially in the ratios of fish oil to MFFAs above 1:5 (Fig. 1). This was due to the SFAs from MFFAs being incorporated to replace the PUFAs in fish oil. This was consistent with Ozturk *et al.*[19], where the higher ratio of free fatty acids to oil in the acidolysis reaction directed the equilibrium of the reaction to product synthesis. Subroto *et al.*[20] also showed that the amount of lauric acid binding to the structured lipids produced by the acidolysis of fish oil with lauric acid continued to increase until the ratio of fish oil and lauric acid was 1:10. The substrate conditions also affected the rate of the enzymatic reactions. The addition of the substrate increased the product conversion during the enzyme loading of the catalytic site. When the amount of the substrate exceeded the enzyme load, the excess substrate had no effect on the product conversion. However, the excess of substrates actually inhibits the lipase activity[21]. Based on Fig. 1, it can be seen that the addition of the MFFAs up to 15 times the amount of the fish oil increased the SFAs but notably decreased the PUFAs even though the desired SLs, the PUFAs at sn-2, remained high. Therefore, the fish oil to MFFAs molar ratio of 1:3 produced the best SLs, where the SFAs increased 1.52 times but the PUFAs did not decrease significantly.

Effect of the fish oil to MFFAs molar ratio on the acylglycerol profile of the SLs: The enzymatic acidolysis of the fish oil with MFFAs resulted in a decrease in the TAG content, while the DAG and MAG contents increased. This was due to the partial hydrolysis of the SLs during the acidolysis reaction. The fish oil used in this experiment was 86.14% TAG, while the DAG and MAG contents were 8.30 and 5.56%, respectively. Figure 2 shows that when the fish oil to MFFAs molar ratio increased to 1:1, the TAG content decreased to 67.86%, while that of MAG and DAG increased to 11.18 and 20,97%, respectively. Further increasing the proportion of MFFAs did not significantly change the acylglycerol profile of the SLs. This finding agrees with Xu *et al.*[22], who stated that the MAG and DAG contents increased in the acidolysis products of rapeseed oil with capric acid. The formation of MAG and DAG during acidolysis can be affected by the system's water content, the acidity of the reactants, the pH, the excessive use of enzymes and changes in the reaction equilibrium[21]. A larger amount of free fatty acids makes the system more acidic and causes acyl migration[23]. In addition, TAG was easier to hydrolyze than DAG and MAG. While MAG was more easily esterified to DAG, the reaction led to the formation of DAG. However, MAG and DAG are emulsifiers. They can increase the enzymatic reaction rate since the enzymatic reaction takes place at the interface[24,25].

Effect of reaction time on the SFA content and the PUFA content in the SLs: The acidolysis reactions of the fish oil with MFFAs ran fast until 6 h. This was indicated by the SFA content in the SLs increasing significantly but a longer reaction time

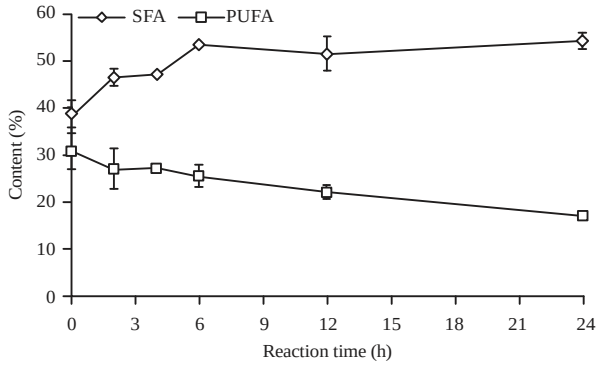

Fig. 3: The effect of reaction time on the SFA content and PUFA content in the SLs
The reaction was performed using 10% immobilized lipase and at molar ratio of fish oil to MFFAs of 1:3 at 40°C

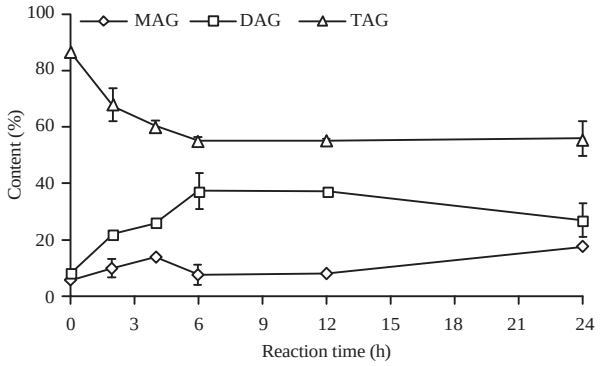

Fig. 4: The effect of reaction time on the TAG content DAG content and MAG content in the SLs
The reaction was performed using 10% immobilized lipase and a molar ratio of fish oil to MFFAs of 1:3 at 40°C

did not significantly increase the SFA content (Fig. 3). This result is in line with that of Kim and Hill[12], where the incorporation of fatty acids during acidolysis increased with an increasing reaction time. Yankah and Akoh[26] reported that the incorporation of caprylic acid into tristearin increased with an increasing reaction time but an equilibrium was reached at 12 h. This was due to several conditions in the reactor, such as the water content, pH and substrate type. This result was also in line with that of Carrin and Crapiste[27], where the rate of the acidolysis reaction of a mixture of sunflower oil and palmitic-stearic acid increased with an increasing reaction time. This research showed that the reaction had reached an equilibrium at 6 h. The reaction time of 6 h increased SFA content 1.39 times from the initial composition of 38.48% to a final concentration of 53.32%, while the PUFA content decreased from 30.64-25.36%. This was because the acidolysis of the fish oil with MFFAs replaced the PUFAs with SFAs. Therefore, a reaction time of 6 h was the optimum time for the enzymatic acidolysis of the fish oil with MFFAs. The addition of a longer incubation time did not significantly increase the number of incorporated of SFAs.

Effect of the reaction time on the acylglycerol profile of the SLs: Figure 4 shows that the acylglycerol profile of the SLs was affected by the reaction time. The TAG content decreased until a reaction time of 6 h and the further increase in the reaction time did not significantly affect it. The -OH bonds in MAG and DAG were more stable than the R-bonds in TAG thus the addition of the reaction time increased the formation of MAG and DAG, while the formation of TAG decreased. In addition, MAG is more polar than DAG, while DAG is more polar than TAG. This allowed MAG to be more easily bound by the lipases whose surfaces are hydrophilic so that MAG could be more easily esterified into DAG. These results agree with those of Xu *et al.*[22], where the MAG and DAG contents in SLs tended to increase and the TAG content decreased compared to the initial composition of rapeseed oil before modification by acidolysis with capric acid. This was due to acyl migration, which causes an imperfect interesterification reaction, where the longer reaction time increased the acyl migration[23]. The TAG content decreased with an increasing reaction time because the reaction was dominated by the partial hydrolysis of TAG to DAG and the esterification of MAG to DAG. The acylglycerol profile of the SLs at a 6 h reaction time, which was the optimum reaction time, contained TAG, DAG and MAG, at 69.45, 22.32 and 8.23%, respectively. The presence of partial glycerides was not a problem because MAG and DAG are food grade. These glycerides, especially DAG loading, have beneficial effects on health[28,29].

Positional distribution of the fatty acid residues in the structured lipid: The structured lipid (SL) from the optimum conditions of acidolysis, namely, the molar ratio of fish oil to MFFAs of 1:3 and a reaction time of 6 h at 40°C, was evaluated for the positional distribution of the fatty acid residues in the SL compared to that in the original oil (before acidolysis). Table 2. shows that the SL contained SFAs at approximately 53.32%, an amount that was increased approximately 1.39 times from that of the original oil at approximately 38.48%. These results indicated that most of the SFAs from the milk fat were incorporated into the fish oil by acidolysis. However, the PUFAs in the SL were lower than those in the original oil but most of the PUFAs were incorporated at the sn-2 position by approximately 26.05%. The PUFAs consisted of EPA (13.20%) and DHA (12.85%). These results indicated that the lipase from *Mucor miehei* only catalyzed sn-1,3 and the acidolysis did

Table 2: Positional distribution of the fatty acid residues in the structured lipid

	Before acidolysis			After acidolysis (SIs)		
Fatty acid	% of total	% of sn-2	% of sn-1,3	% of total	% of sn-2	% of sn-1,3
Capric	0.25±0.04	ND	0.33±0.06	1.74±0.14	ND	2.60±0.56
Lauric	0.74±0.12	0.33±0.07	0.83±0.41	3.29±0.13	ND	4.94±0.19
Miristic	10.47±0.70	5.59±0.68	12.18±0.71	10.90±0.84	8.23±1.36	12.24±1.93
Palmitic	22.48±1.46	22.48±1.96	22.48±1.21	28.68±0.26	24.87±1.20	30.59±0.99
Palmitoleic	13.63±1.15	9.89±1.21	15.50±2.83	6.59±0.24	6.04±0.42	6.87±0.57
Stearic	5.13±0.49	7.09±1.59	4.16±0.06	8.71±0.62	10.36±0.22	7.88±1.05
Oleic	17.25±0.18	22.73±0.8	14.51±0.13	14.73±1.55	24.46±0.56	9.86±0.55
EPA	18.32±2.5	18.93±1.43	18.76±1.59	12.93±1.26	13.20±0.29	12.80±1.74
DHA	12.32±1.28	12.93±1.69	11.26±1.7	12.43±0.57	12.85±0.09	12.22±0.91
∑ SFA	38.48	35.49	39.97	53.32	43.46	58.25
∑ PUFA	30.64	31.88	30.02	25.36	26.05	25.02

Each value is presented as the mean±standard deviation (n=2), ND: Not detected, SFA: Saturated fatty acid, PUFA: Polyunsaturated fatty acid, EPA: Eicosapentaenoic acid, DHA: Docosahexaenoic acid

not change the PUFAs at the sn-2 position. According to Rodrigues and Fernandez-Lafuente[30], the lipase from *Mucor miehei* has a specific activity at the sn-1,3 position. This was confirmed by the SFAs in the SL, which were incorporated at sn-1,3 at approximately 58.25%, which was higher than in the original oil. However, the fatty acid composition at the sn-2 position was slightly different than that of the original oil. These differences can be attributed to the acyl migration during acidolysis or interesterification[23] but are generally consistent with the sn-1,3 specificity of the lipase from *Mucor miehei*.

The results showed that the acidolysis reaction of the fish oil with MFFAs using the lipase from *Mucor miehei* produced SLs whose outer portion was filled by SFAs, while the PUFAs remained in the middle (sn-2). The SL had a good nutritional value because the PUFAs were in sn-2, while the SFAs, including the MCFAs, were on the outside and thus can be easily metabolized. The SLs that contained SFAs on the outside were also more resistant to oxidation[5].

CONCLUSION

The increasing fractions of MFFAs and reaction times increased the incorporation of SFAs. The optimum conditions for acidolysis were a ratio of fish oil to MFFAs of 1:3 and a reaction time of 6 h at 40°C. In these conditions, a good SL was produced, where sn-2 comprised EPA and DHA at 13.20 and 12.85%, respectively and the total PUFA was 26.05%, where sn-1,3 comprised SFAs (namely, capric, lauric, myristic, palmitic and stearic acids) at 58.25%. The SL had an acylglycerol profile containing triacylglycerol (TAG), diacylglycerol (DAG) and monoacylglycerol (MAG) at 69.45%, 22.32 and 8.23%, respectively.

SIGNIFICANCE STATEMENT

Fish oil is rich in PUFAs, especially EPA and DHA but PUFAs are easily oxidized and only good when in the sn-2 position. Therefore, sn-1,3 of fish oil needs to be replaced with SFAs that are resistant to oxidation. Many researchers have made SLs from fish oil and saturated fatty acids but the use of SFAs from milk fat remains an unexplored area of research. In this study, we synthesized SLs by the enzymatic acidolysis of fish oil with free fatty acids from milk fat called milk fat fatty acids (MFFAs) using a specific lipase from *Mucor miehei*. This research is useful for providing an SL that has the potential to fortify high-nutrition dairy products.

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